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14. ABSTRACT

The Stat3 pathway and the hypoxia sensing pathway are both upregulated in prostate cancer. Stat3 is a specific regulator of pro-carcinogenic inflammation and represents a promising therapeutic target for converting cancer-promoting inflammation to anti-tumor immunity. HIF-1αwhich mediates the cellular response to hypoxia, has been demonstrated to be overexpressed in many human cancers and is associated with poor prognosis and treatment failure in clinic. To develop a potent strategy to increase therapeutic efficacy and reduce drug resistance in prostate cancer therapy, we combined two anti-cancer agents: T40214 (a p-Stat3 inhibitor) and JG244 (a HIF-1α inhibitor) together to treat nude mice bearing human prostate tumor (DU145), and immunocompetent mice (C57BL/6) bearing murine prostate tumor (TRAMP-C2). The results showed that targeting p-Stat3 and HIF-1α together greatly promoted drug efficacy as compared to either agent used alone. Western blots and TUNEL assays provided evidence that, compared with each agent alone, the combination treatment dramatically increased apoptosis in tumors and enhanced drug efficacy, suggesting that the combination treatment including a HIF-1α/2α inhibitor not only has therapeutic efficacy in targeting HIF-1α/2α but can also reduce hypoxia-induced drug resistance and enhance drug efficacy for the combined therapeutic agents, potentially making standard prostate cancer treatments more effective.

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INTRODUCTION.

A1.Significance. The goal of this proposal is to develop a novel therapeutic strategy for treating prostate cancer using a combination of newly developed agents that target Stat3 and HIF-1α. Prostate cancer is the most frequently diagnosed cancer among men in the United States and is second only to lung cancer as a cause of cancer death. In the year 2008, carcinoma of the prostate accounted for 186,320 new cancer cases and 28,660 deaths in the United States. Prostate cancer initially occurs as an androgen-dependent tumor and patients with metastatic disease respond initially to androgen–ablation therapy. However, when the disease recurs it is often insensitive to hormonal manipulation. Salvage therapy for hormone refractory metastatic disease includes chemotherapeutic agents which can have undesirable side effects and produce relatively short survival periods. In fact, median survival is only 18.9 months even with the most active chemotherapeutic agents (1). Therefore, novel therapies, including agents with different therapeutic mechanisms of action and novel molecular targets, are urgently needed to treat prostate cancer patients.

Two pathways which are upregulated in prostate cancer are the Stat 3 pathway and the hypoxia sensing pathway. Signal transducer and activator of transcription 3 (Stat3) has been identified as an important target for cancer therapy, since it participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors (Bcl-x$_L$, Bcl-2, Mcl-1, and survivin), cell-cycle regulators (cyclin D1 and c-*myc*), and inducers of angiogenesis (VEGF) (2). Stat3 is constitutively activated in more than 80% of prostate cancers (3). Also, Stat3 is constitutively activated in immune cells in the tumor microenvironment (4). Targeting phosphorylated Stat3 (p-Stat3) is expected to decrease the tumor cell survival, decrease angiogenic potential and cell proliferation.

Within tumors, the availability of O_2 and nutrients is limited by competition among proliferating cells, and diffusion of metabolites is inhibited by high interstitial pressure (5). Hypoxia, which serves as a critical factor for both physiological and pathological angiogenesis, is an important factor in the progression and therapeutic resistance of many human cancers (6). To date, more than 70 putative hypoxia-inducible genes have been found to be directly regulated by HIF-1 (7,8). Under normoxic conditions the cellular half-life of HIF-1α is < 5 minutes, as the protein is rapidly degraded by the ubiquitin-proteasome system. Under hypoxic conditions, HIF-1α is stabilized by the absence of posttranslational prolyl hydroxylation at residues P402 and P564 (in human HIF-1α) (9). HIF-1α has been demonstrated to be overexpressed in many human cancers, including colon, breast, gastric, lung, skin, ovarian, prostate, renal and pancreatic carcinomas (7). Overexpression of HIF-1α in human cancers, which results from intratumoral hypoxia and genetic alternations, has been associated with poor prognosis and treatment failure in a number of cancers (7). Targeting HIF-1α could constitute a novel and potent cancer therapy.

A2. Hypothesis and aims. Drug development has moved into targeting molecules based on their important biological function in cancer (10). However, selective inhibition of a target molecule can result in unexpected consequences, e.g. rapid development of resistance as a single molecule is inhibited with high selectivity; loss of drug effect after prolonged use; reduced delivery of an anticancer agent to all regions of the tumor; and a change in micro-environmentthat reduces drug

response. In order to promote therapeutic efficacy and revolutionize the treatment of prostate cancer, we propose a novel combinational strategy for treatment of prostate cancer. We hypothesize that activation of Stat3 and HIF-1 strongly influence progression of prostate cancer, using a strategy that targets both Stat3 and HIF-1α could produce an effective treatment for prostate cancer. Targeting Stat3 is expected to decrease survival of tumor cells, inhibit tumor angiogenesis and activate immune cells in the tumor stroma. Inhibition of HIF-1α not only suppresses tumor growth, but also alters tumor micro-environment, which could increase drug efficacy and decrease drug resistance. Targeting both p-Stat3 and HIF-1α using T40214 and JG244 could increase tumor response as compared to blocking activation of either molecule alone. Alternating administration of the agents may provide a mechanism to reduce drug resistance and treatment failure. We will explore our hypothesis via two specific aims:

<u>**Specific Aim1:**</u> To develop a strategy for treating prostate cancer using a combination of newly developed agents (T40214 and JG244) that target Stat3 and HIF-1α in cultured cells.

<u>**Specific Aim2:**</u> To determine the in vivo drug efficacy of combination treatment**.**

BODY AND KEY RESEARCH ACCOMPLISHMENTS (see details in attached paper**).**

1. **Inhibitors of p-Stat3 and HIF-1α.** Here we have employed two agents T40214 and JG244 to target p-Stat3 and HIF-1α, respectively, and block DU145 prostate tumor growth. The AFM (atomic force microscopy) results demonstrated that the size of ODN/PEI complex is about 25nm and the zeta potential showed the surface charge of ODN/PEI complex. Also, we performed the assays of delivering ODN/PEI complex in prostate cancer cells (DU145) using fluorescent labeled T40214 and JG244. The results demonstrated that T40214 and JG244 were well delivered inside cells and then entered into nucleus. This delivery system significantly increases the drug efficacy of these agents (14,15).

2. **Suppressing the levels of p-Stat3 and HIF-1α.** The western blots showed that the IC_{50}s of inhibition of HIF-1α and HIF-2α by JG244 are ~ 2.4 to 2.7μM while T40214 showed no inhibition of HIF-1α and HIF-2α. In contrast, T40214 inhibited IL-6-induced p-Stat3 activation in DU145 cells and its IC_{50} is estamited as 3.3μM, while JG244 did not show any inhibition of p-Stat3. The results provide evidence that T40214 and JG244 selectively inhibit their respective targets, p-Stat3 and HIF-1α/2α, in both human and murine prostate cancer cells.

3. **Induction of ap optosis in prostate cancer cells.** Inducing apoptosis in cancer cells is an important factor for anti-cancer agents. Flow cytometry of propidium iodide-stained DU145 cells was employed to identify the population of sub-G1 (SG1) cells, which manifest DNA damage associated with apoptotic or necrotic cell death. The results demonstrated that compared with the SG1 fraction of control cells (0.8%), no apoptosis was induced in the cells treated by PEI alone (1.2%) or ns-ODN/PEI (0.5%), which is a 20mer non-G-rich ODN used as a control. The percentage of cells in SG1 increased to 18.1% for T40214-treated and 21.2% for JG244-treated DU145 cells. However, the combined treatment mixing T40214 and JG244 together induced the percentage of cells in SG1 to 35.2%, showing that the treatment with conbined two inhibitors together dramatically induced apoptosis in prostate cancer cells.

4. *In vivo* **anti-tumor efficacy.** Tests of *in vivo* anti-tumor efficacy were performed by administration of the two agents, T40214 or JG244, every three days alone or alternately via intravenous injection. Nude mice with established (~150mm^3) metastatic prostate tumors

(DU145) were randomly assigned to 5 groups: (i) untreated; (ii) PEI (2.5mg/kg); (iii) T40214/PEI (10mg/kg+2.5mg/kg); (iv) JG244/PEI (10mg/kg+2.5mg/kg); and (v) T40214/PEI and JG244/PEI (10mg/kg+2.5mg/kg) alternately (e.g. T40214/PEI on day1, day7, day13, etc., and JG244/PEI on day4, day10, day17, etc.). Tumor volume and body weight were measured every three days. Over 31 days of drug treatment, the mean tumor volume in control mice and PEI-treated mice increased 5.8 and 5.2 fold and mean body weights decreased 5-7%. The mean tumor volumes in mice treated with T40214/PEI and JG244/PEI alone increased 3.1 and 2.5 fold, respectively. The mean tumor volume in mice treated by alternating JG244/PEI and T40214/PEI increased only 1.5 fold. The mean body weights of the drug-treated groups increased about 5-10%, showing the two agents were not overtly toxic and were well tolerated by animals. Statistical analysis indicated that, compared with untreated tumors, the growth of the tumors treated by T40214 ($p<0.05$) or JG244 ($p<0.01$) or the combination of T40214 with JG244 ($p<0.002$) were all significantly suppressed. Moreover, compared with T40214 alone, T40214 and JG244 given sequentially showed a significant increase in drug efficacy ($p<0.05$).

To investigate whether the efficacy of T40214 and JG244 would be affected by the immune system, immunocompetent mice (C57BL/6) bearing murine prostate tumors (TRAMP-C2) were employed for *in vivo* drug tests. The sequence of Stat3 protein in murine cells is the same as that in human cells (16). Also, a comparison between the murine and human HIF-1α protein sequence revealed 95%, 99%, and 83% identity in the bHLH, PAS, and variable domains, respectively (17). When tumor size reached ~150 mm^3, the mice were randomly assigned to various groups: (i) untreated (control); (ii) PEI alone (2.5mg/kg); (iii) ns-ODN/PEI as a control oligo (10mg/kg+2.5mg/kg); (iv) paclitaxel, a chemotherapeutic drug, as a positive control (10mg/kg); (v) T40214/PEI (10mg/kg+2.5mg/kg); and (vi) combination treatment mixing T40214 with JG244 ((5+5)mg/kg+2.5mg/kg). The mice were treated every other day by intraperitoneal injection for three weeks (paclitaxel was given every four days due to its toxicity). Over 21 days of drug treatment, the results demonstrated that the mean tumor volumes of control, PEI-treated, ns-ODN/PEI-treated, and paclitaxel-treated mice increased 9.4, 9.4, 10.2, and 8.0 fold, respectively. The mean tumor volumes of the mice treated by T40214 alone and T40214/JG244 combined increased 5.6 ($p<0.09$) and 3.1 ($p<0.03$) fold, showing that combining T40214 and JG244 together significantly suppressed the murine prostate tumor growth in immunocompetent mice.

5. **Apoptosis induced by T40214 and JG244**. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) is a standard method for detecting DNA fragmentation by labeling the terminal ends of nucleic acids that result from apoptotic signaling cascades. Here we performed TUNEL assays in the harvested tumors using the TumorTACS *In Situ* Apoptosis Detection Kit (Trevigen, Inc. MD) according to the manufacture's instruction. The kit-stain generated dark brown precipitates in cleaved DNA fragments, which correspond to apoptotic cells. The results from DU145 tumors demonstrated that there are few apoptotic cells in untreated (3%) and PEI-treated tumors (8%), and many apoptotic cells in T40214/PEI-treated (43%) or JG-244/PEI-treated tumors (39%). The combination treatment further dramatically promoted apoptosis in the prostate tumors (78%). Similar results were also observed in TRAMP-C2 tumors. Compared with apoptosis induced by T40214 (35%), treatment with T40214 plus JG244 greatly enhanced apoptosis in the tumors (56%). Statistical analysis indicated that in both DU145 and TRAMP-C2 tumors the indexes of apoptotic cells between untreated and T40214/JG244-treated tumors or between PEI-treated and T40214/JG244-

treated tumors are significance (p<0.001). Compared with apoptotic cells induced by T40214 or JG244 alone, the combined treatment greatly increased apoptosis in DU145 (p<0.006) and TRAMP-C2 tumors (p<0.008). These results provided solid evidence that compared with the tumors treated by Stat3 inhibitor or HIF-1α inhibitor alone, combination treatment with inhibiters of both p-Stat3 and HIF-1α can significantly induce cell death in prostate tumors.

CONCLUSION.

The Stat3 pathway and the hypoxia sensing pathway are both upregulated in prostate cancer. Stat3 is a specific regulator of pro-carcinogenic inflammation and represents a promising therapeutic target for converting cancer-promoting inflammation to anti-tumor immunity. HIF-1α, which mediates the cellular response to hypoxia, has been demonstrated to be overexpressed in many human cancers and is associated with poor prognosis and treatment failure in clinic. To develop a potent strategy to increase therapeutic efficacy and reduce drug resistance in prostate cancer therapy, we combined two anti-cancer agents: T40214 (a p-Stat3 inhibitor) and JG244 (a HIF-1α inhibitor) together to treat nude mice bearing human prostate tumor (DU145), and immunocompetent mice (C57BL/6) bearing murine prostate tumor (TRAMP-C2). The results showed that targeting p-Stat3 and HIF-1α together greatly promoted drug efficacy as compared to either agent used alone. Western blots and TUNEL assays provided evidence that, compared with each agent alone, the combination treatment dramatically increased apoptosis in tumors and enhanced drug efficacy. Threrfore, based on our studies, the treatment using HIF-1α inhibitor (JG244) combined with Stat3 inhibitor (T40214) dramatically increased apoptosis in prostate tumors and significantly suppressed tumor growth. Previous studies demonstrated that overexpression of HIF-1α contributes to resistance to both radiation therapy, and chemotherapy and is associated with treatment failure and increased patient mortality (11-13). Our results showed that combination treatment targeting p-Stat3 and HIF-1α can increase tumor response as compared to either agent alone. Alternating the two agents provides a novel mechanism to reduce drug resistance and treatment failure. In addition, inhibition of HIF-1α can potentially reduce the hypoxia influence on cancer cell survival. Our results provide evidence that the combination treatment including a HIF-1α/2α inhibitor not only has therapeutic efficacy in targeting HIF-1α/2α but also can reduce hypoxia-induced drug resistance and enhance drug efficacy.

REFERENCES.

1. Tannock IF, deWit R et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for athe Cognized Federal Agency field-DHHS, Henry Williams, 214-767-3600 dvanced prostate cancer. N Engl J Med. 2004; 351:1502-12.
2. Yu H, Jove R. (2004) The STATS of cancer-new molecular targets come of age. *Nature Rev/Cancer* 4, 97-105.
3. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, Muro-Cacho C, Livingston S, Karras J, Pow-Sang J, Jove R. (2002) Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Research* 62, 6659-66.

4. Yu H, Kortylewski M, pardoll D. (2007) Crosstalk between cancer and immune cells: role of Stat3 in tumor microenvironment. *Nat Review Immunol* 7, 41-51.
5. Fukumura, D. and Jain, R.K. (2007) Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. *Microvasc Res*, **74**, 72-84.
6. Harris, A.L. (2002) Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer*, **2**, 38-47.
7. Semenza, G.L. (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3**, 721-732.
8. Hirota, K. and Semenza, G.L. (2006) Regulation of angiogenesis by hypoxia-inducible factor 1. *Crit Rev Oncol Hematol*, **59**, 15-26.
9. Kaelin, W.G., Jr. and Ratcliffe, P.J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*, **30**, 393-402.
10. Hambley TW. Anticancer drug development heading in the right direction? (2009) Cancer res. 69, 1259-62.
11. Chun YS, Adusumilli PS, Fong Y. (2005) Employing tumor hypoxia for oncolytic therapy in breast cancer. *J Mammary Gland Biol Neoplasia* **10:** 311-318.
12. Fontanini G, Faviana P, Lucchi M, et al. (2002) A high vascular count and overexpression of vascular endothelial growth factor are associated with unfavourable prognosis in operated small cell lung carcinoma. *Br J Cancer* **86:** 558-563.
13. Ishigami SI, Arii S, Furutani M, et al. (1998) Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. *Br J Cancer* **78:** 1379-1384.
14. Weerasinghe P, Li Y, Guan Y, Zhang R, Tweardy DJ, Jing N. (2008) T40214/PEI complex: a potent therapeutics for prostate cancer that targets STAT3 signaling. *Prostate* **68:** 1430-1442.
15. Guan Y, Ramasamy Reddy K, Zhu Q, et al. (2009) G-rich Oligonucleotides Inhibit HIF-1alpha and HIF-2alpha and Block Tumor Growth. *Mol Ther*.
16. Pietra LD, Bressan A, Pezzotti AR, Serlupi-Crescenzi O. (1998) Highly conserved amino-acid sequence between murine STAT3 and a revised human STAT3 sequence. *Gene* **213:** 119-124.
17. Luo G, Gu YZ, Jain S, et al. (1997) Molecular characterization of the murine Hif-1 alpha locus. *Gene Expr* **6:** 287-299.

REPORTABLE OUT COMES.

Publications:
1. Reddy KR, Guan Y, Qin G, Zhou Z, **Jing N**. "Combined treatment targeting HIF-1   and Stat3 is a potent strategy for prostate cancer therapy" *Prostate* (2011).
2. **Jing N**. "Rational design of DNA anti-cancer agent that target signal tranducer and activator of transcription (Stat3) for cancer therapy" *Book: SIGNALLING MECHANISM and MOLECULAR TARGETS IN CANCER* (2011) (in press).

Meeting Abstracts.
1. Naijie Jing:
    Title: Establishing a rational drug desing system to develop a novel anti-cancer agnet.
    ABSTRACT: A key step to discover or develop a new anti-cancer agent is to establish a rational drug design system. Based on an established drug design system in my lab, we have developed two anti-cancer agents: (i) T40214 as a phosphorylated Stat3 inhibitor

was recently accepted by RAID program, NCI, as a promising candidate of anti-cancer agent. RAID will help us to get critical data for FDA approval; (ii) JG-ODN as a HIF-1α inhibitor has been recently developed as a candidate for cancer therapy. Using our developed anti-cancer agents as exemplars, I will introduce how to establish a rational drug design system for developing a new candidate of anti-cancer drug, which includes: structure based drug design (or screen); *in vitro* drug active tests; establishing a structure-activity relationship (SAR) for rational drug design or optimization; assay of *in vivo* drug efficacy; determining the drug mechanism and selectivity in tumor tissues; and employing a microarray to identify the oncogene suppression if necessary.

2. Reddy KR, Guan Y, Qin G, Zhou Z, Jing N
   Title: A Novel Combination Treatment that Targets Stat3/AR and HIF-1α for Prostate Cancer Therapy
   ABSTRACT: The Stat3 pathway and the hypoxia sensing pathway are both upregulated in prostate cancer. Stat3 is a specific regulator of pro-carcinogenic inflammation and represents a promising therapeutic target for converting cancer-promoting inflammation to anti-tumor immunity. HIF-1alpha (HIF-1a)which mediates the cellular response to hypoxia, has been demonstrated to be overexpressed in many human cancers and is associated with poor prognosis and treatment failure in clinic. To develop a potent strategy to increase therapeutic efficacy and reduce drug resistance in prostate cancer therapy, we combined two anti-cancer agents: T40214 (a p-Stat3 inhibitor) and JG244 (a HIF-1a inhibitor) together to treat nude mice bearing human prostate tumor (DU145), and immunocompetent mice (C57BL/6) bearing murine prostate tumor (TRAMP-C2). The results showed that targeting p-Stat3 and HIF-1a together greatly promoted drug efficacy as compared to either agent used alone. Western blots and TUNEL assays provided evidence that, compared with each agent alone, the combination treatment dramatically increased apoptosis in tumors and enhanced drug efficacy, suggesting that the combination treatment including a HIF-1a/2a inhibitor not only has therapeutic efficacy in targeting HIF-1a/2a but can also reduce hypoxia-induced drug resistance and enhance drug efficacy for the combined therapeutic agents, potentially making standard prostate cancer treatments more effective.

3. Payment list. Jing N; Guan Y; Reddy KR; Tweardy DJ.

ABSTRACT OF PROJECT II.

Our main goal in this project is to develop a novel anti-cancer drug for prostate cancer therapy. Two pathways: Stat3 and HIF-1 are demonstrated to be critical targets for cancer therapy. Signal transducer and activator of transcription 3 (Stat3) participates in oncogenesis through the up regulation of genes encoding anti-apoptosis, cell-cycle regulators, and inducers of angiogenesis. Also, Stat3 suppresses anti-tumor immune responses and promotes inflammation-induced cancer, making it an attractive target. HIF-1 (hypoxia-inducible factor-1) mediates the cellular response to hypoxia and activates the transcription of genes that are involved angiogenesis, cell survival, glucose metabolism and invasion. HIF-1α was demonstrated to overexpress in many human cancers, including colon, breast, and gastric, lung, and skin, ovarian, prostate, renal and pancreatic carcinomas. Overexpression of HIF-1α, which results from intratumoral hypoxia and

genetic alternations, has been associated with poor prognosis and treatment failure in a number of cancers.

Our previous results demonstrated that targeting both p-Stat3 and HIF-1α together could improve tumor response to either agent alone, and reduce drug resistance and treatment failure. Recently, in collaboration with a group in Peking University (China), we isolated several novel molecules from Chinese herbal medicines. After activity tests of these molecules, we found one compound, TEL, that is a derivative of *hypocrellin* (a nature product from a Chinese medicinal nerb), selectively targets both HIF-1α and p-Stat3, significantly inhibit activation of HIF-1α and p-Stat3, and block the expression of both HIF-1α and p-Stat3 down-regulated oncogenes (e.g. Bcl2, VEGF, and others) in cancer cells, showing that it has great potential to be anti-cancer agents. Therefore, we propose that TEL as an inhibitor of both HIF-1α and p-Stat3 from could have great potential to make cancer treatments more effective and improve survival of patients with metastatic disease. In this project, we designed a research plan with three aims to expand these efforts. (1) To isolate and synthesize additional novel compounds from Chinese herbal medicines for testing in HIF-1α and Stat3 inhibition. (2) Determine drug activity, function and mechanism of action of the molecules obtained in Aim1. (3) Determine drug efficacy of TEL in xenograft models bearing breast cancer tumors.

Combined Treatment Targeting HIF-1α and Stat3 is a Potent Strategy for Prostate Cancer Therapy

Kavitha Ramasamy Reddy,[1] Yongli Guan,[1] Guoting Qin,[3] Zhou Zhou,[1] and Naijie Jing[1,2]*

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BACKGROUND. The Stat3 pathway and the hypoxia-sensing pathway are both up-regulated in prostate cancer. Stat3 is a specific regulator of pro-carcinogenic inflammation and represents a promising therapeutic target. Hypoxia-inducible factor-1 (HIF-1)α, which mediates the cellular response to hypoxia, has been demonstrated to be over-expressed in many human cancers and is associated with poor prognosis and treatment failure in clinic. To develop a potent strategy to increase therapeutic efficacy and reduce drug resistance in prostate cancer therapy, we combined two anti-cancer agents: T40214 (a p-Stat3 inhibitor) and JG244 (a HIF-1α inhibitor) together to treat nude mice bearing human prostate tumor (DU145) and immunocompetent mice (C57BL/6) bearing murine prostate tumor (TRAMP-C2).

METHODS. We employed in vitro and in vivo assays, including Western blots, cell cycle analysis, immunohistochemistry, TUNEL and xenograft models to determine the drug efficacy and mechanism of combination treatment of T40214 and JG244.

RESULTS. We found that compared to treatment by T40214 or JG244 alone, the combination treatment using T40214 and JG244 together significantly suppressed growth of human or murine prostate tumors. Also, compared with apoptotic cells induced by T40214 or JG244 alone, the combined treatment greatly increased apoptosis in DU145 ($P < 0.006$) and TRAMP-C2 tumors ($P < 0.008$).

CONCLUSIONS. Our results suggested that combination treatment including a HIF-1α/2α inhibitor not only has therapeutic efficacy in targeting HIF-1α/2α, but also could reduce the hypoxia-induced drug resistance to other therapies (e.g., T40214) and enhance drug efficacy. This approach could make prostate cancer treatments more effective. *Prostate*
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KEY WORDS: Stat3; HIF-1α; Prostate cancer therapy; T40214; JG244

INTRODUCTION

Two pathways, which are up-regulated in prostate cancer are the signal transducer and activator of transcription 3 (Stat3) and the hypoxia sensing pathway. Stat3 activation is essential to the growth and survival of cancer cells [1,2]. When stimulated by cytokines [3,4], Stat3 is activated upon phosphorylation on tyrosine residue Y705 [5]. Tyrosine phosphorylation induces formation of a parallel dimer [6,7], which then translocates into the nucleus where it binds to DNA-response elements in the promoters of target genes and activates transcription. The activated Stat3 mediates the cancer-promoting properties, such as angiogenesis and anti-apoptosis. Persistently activated Stat3 has been found in many human cancers (e.g., prostate, breast, lung, head and neck, and pancreas, etc.) [8,9]. Recent studies have demonstrated that Stat3 is a specific regulator of pro-carcinogenic inflammation and is constitutively activated not only in tumor cells but also in immune cells in the tumor

microenvironment [10]. The persistent activation of Stat3 mediates tumor-promoting inflammation and the suppression of anti-tumor immunity [11]. Therefore, Stat3 represents a promising therapeutic target for converting cancer-promoting inflammation to anti-tumor immunity. Targeting phosphorylated Stat3 (p-Stat3) is expected to decrease tumor cell survival and angiogenesis, promote anti-immunity and could constitute a potent cancer therapy.

Hypoxia, a constituent of structurally and functionally disturbed microenvironment, is a hallmark of solid tumors and is associated with tumor progression, metastases, and poor patient survival. Hypoxia-inducible factor-1 (HIF-1), which mediates the cellular response to hypoxia, activates the transcription of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism, and invasion. HIF-1α is the key protein that controls the amount of HIF-1 dimer and transcription of the hypoxia-inducible genes [12]. HIF-1 protein is a heterodimer consisting of two subunits: HIF-1α and HIF-1β. Under normoxia, prolyl hydroxylases hydroxylate the prolyl residues of HIF-1α at amino acids P402 and P564, which are then recognized by VHL (Von Hippel-Lindau) and targeted to the ubiquitin proteasome pathway. An additional hydroxylation at N803 blocks the binding of p300 and Creb-binding protein (CBP) to HIF-1α and inhibits HIF-1-mediated gene transcription. Under hypoxia, HIF-1α is not hydroxylated and not degraded. The unmodified protein then dimerizes with HIF-1β. When N803 is not asparaginyl-hydroxylated, p300 and CBP can bind to HIF-1α, allowing transcriptional activation of HIF-1 target genes, which are involved in many cell processes including glucose metabolism, erythropoiesis, angiogenesis, anti-apoptosis, metastasis, and other functions [12,13]. Clinically, HIF-1α over-expression has been shown to be a marker of highly aggressive disease and has been associated with poor prognosis and treatment failure in a number of cancers [14,15]. HIF-1α mediates hypoxia-induced increase of vascular endothelial growth factor (VEGF) expression in tumors leading to aggressive tumor growth [12]. VEGF is a potent angiogenesis promoter and is over-expressed in many human cancers, including human prostate cancer. It enhances migration and proliferation of endothelial cells to stimulate blood vessel formation that in turn promotes tumor growth and metastatic spread [16].

Previously, we have developed the G-rich oligodeoxynucleotide (ODN) T40214 as an inhibitor of phospho-Stat3. T40214 as a potent inhibitor of Stat3 has several unique features, such as selectively targeting the phosphorylated Stat3 and promoting apoptosis, reducing angiogenesis and cell proliferation, and

significantly suppressing tumor growth in animal models bearing human prostate, breast, head and neck, and lung cancers [17–22]. Subsequently, based on rational drug design, we have laid the initial groundwork to develop a novel inhibitor of HIF-1α JG-ODN. The two compounds, JG243 and JG244, selectively inhibited the activity of HIF-1 and HIF-2 and suppressed the expression of HIF-1-regulated proteins (e.g., VEGF, Bcl-2, and Bcl-X$_L$) [23] but do not disrupt the expression of p300, HIF-1β, and p53 [15]. The results demonstrated that targeting HIF-1α by JG-ODN dramatically suppressed solid tumor growth in xenograft models (e.g., prostate, breast, and pancreatic tumors) and that T40214 and JG-ODN mediate their anti-cancer effects by selectively targeting phospho-Stat3 and HIF-1α, respectively [23]. T40214 and JG244 are both G-rich ODNs and form G-quartet structures. Polyethylenimine (PEI) was selected to be a carrier for G-rich ODN, facilitating its delivery to target cells based on endocytosis. This delivery system significantly increases G-rich ODN drug efficacy [21,23]. In this report, we employ the two developed inhibitors, T40214 and JG244, to treat metastatic prostate cancer in vivo. Our results demonstrate that a combination treatment targeting both p-Stat3 and HIF-1α together significantly induces cancer cell apoptosis and enhances in vivo drug efficacy as compared with single agents blocking activation of either p-Stat3 or HIF-1α molecule alone.

MATERIALS AND METHODS

Materials

The antibodies used: mouse phospho Stat3 (Tyr 705), pStat3 (D3A7), stat3 and mouse IgG HRP-conjugated (Cell Signaling Technology), HIF-1α (BD Biosciences), VEGF, PSA, and AR (Santa Cruz Biotechnology), FLAG, β-actin, vinculin (Sigma), and rabbit IgG HRP conjugated (Thermo Scientific). The G-rich ODNs: T40214 (GGGCGGGCGGGCGGGC) and JG244 (GGCGGGTAGGCGGG) and non-specific oligonucleotide (ns-ODN, TGCCGGATCAAGAGC-TACCA) were synthesized and purified by Midland Certified Reagent Company and used without further chemical modifications. PEI, ~25 kDa polymer was purchased from Aldrich. Human DU145 Prostate cancer cells were purchased from ATCC and grown in EMEM medium, respectively. TRAMP-C2 mouse prostate cancer cells were the gift of Dr. David Spencer (Baylor College of Medicine), and were cultured in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 units/ml streptomycin and maintained in a 37°C CO$_2$ incubator.

Atomic-Force Microscopy (AFM)

AFM imaging of the nanoparticles was performed using a MultiMode Nanoscope IIIa AFM (Digital Instruments Inc., Santa Barbara, CA). Images were acquired in tapping mode using a silicon nitride cantilever (MikroMasch, San Jose, CA) with a resonance frequency of ~130 KHz and a nominal force constant of ~1.75 N/m.

Zeta Potential and Size of JG244/PEI complex

A solution of JG244 (5 µg/µl) in sterile H_2O was mixed with a solution of PEI in sterile H_2O at a ratio of ODN/PEI as 1:2. Immediately after the mixing, the mixture was rapidly injected into a pre-rinsed zeta cell and the zeta potential and size of the complexes was monitored over a period of 30 min, using a Zeta Sizer Nanoseries (Malvern Instruments).

Assay of PEI Delivery

DU145 cells ($5–6 \times 10^4$) were seeded per well of a 24-well plate coated with nanobeads and grew 20 hr in EMEM with 10% FBS per well. Then the cells were treated with the T40214/PEI and JG244/PEI complexes. The complexes were composed of fluorescence labeled and non-labeled T40214 and JG244 (at ratio of 1:9) with PEI at ratio of ODN/PEI as 1:2. The ODN/PEI complexes were added into each well with growth medium without FBS (to avoid interaction) and incubate 3 hr in 37°C CO_2 incubator. After the incubation, cells were washed once with PBS and grew over night for detection. Then the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilized with 0.1% Triton X-100 in PBS and counterstained with DAPI (1:1,000, Molecular Probes Inc., Eugene, OR). The Intracellular delivery of the G-quartet ODN was detected by intracellular fluorescence under a fluorescent microscope (200X, Olympus IX81, Olympus America Inc., Center Valley, PA).

Drug Treatment in Cell Lines

DU145 prostate cancer cells at 50–60% confluence in 100-mm tissue culture plates were treated without or with IL-6 at 50 ng/ml concentration for 30 min. Then cells were washed twice with serum-free medium and added with the complex of T40214/PEI or JG244/PEI at 1.0, 2.5, and 5.0 µM. After addition of the oligos, cells were kept in an incubator at 37°C for 3 hr. The cells were then washed three times with serum-free medium and incubated in a hypoxia chamber with 1% O_2, 5% CO_2, and 94% N_2 at 37°C overnight, followed by protein extraction for Western blot analysis to determine drug activities.

Western Blots

Cells were lysed in cell lysis buffer (Cell Signaling) supplemented with protease inhibitor cocktail (Sigma) and phosphatase cocktail inhibitor (Sigma). Total protein (80 µg) was resolved in 10%SDS–PAGE gels, transferred to Hybond-ECL nitrocellulose membrane, blocked with 5% non-fat skim milk, and probed with specific antibodies against tyrosine–phosphorylated STAT3 (p-Stat3), total STAT3 (T-Stat3), HIF-1α, VEGF, or β-actin (or vinculin). Prostate tumors were harvested after drug treatments, homogenized, and lysed in cell lysis buffer containing protease cocktail inhibitor and phosphatase cocktail inhibitors I and II. Tumor tissue proteins (50 µg) were resolved on SDS–PAGE gels and probed by specific antibodies, as described above. The bands were quantified using a Personal Densitometer Scanner (version 1.30) and ImageQuant software (version 3.30 (GE Healthcare/ Amersham Biosciences).

Serum Stability Assay

Samples of whole blood were taken from mice. Serum was collected by centrifugation at 1,500 rpm for 15 min. Fluorescence-labeled GQ-ODN T40214 and JG244 were mixed to a final concentration of ~0.05 µg/µl with 200 µl of mouse serum and then were immediately incubated at 37°C. Aliquots of 10 µl were taken starting from 0, 0.5, 1, 2, 4, 6, 12, 24, 48, 72, and 96 hr. Reactions were terminated by addition of 10 µl of $1\times$ formamide containing loading buffer (Ambion) and subsequent freezing at −80°C. For analysis, the samples were heated to 60°C for 3 min and full length and digested oligomers were then separated on a denaturing 15% polyacrylamide gel (7 M urea). The gels were exposed under UV light and photographed by ChemiDoc XRS Plus (Bio-Rad Laboratories). Densitometric calculations were done with Chemidoc volume analysis. The volume density of the major band corresponding to intact ODN was calculated with 5% error deviation. The density of ODN at time 0 was set as a reference (100%). Relative values for the other time-points were calculated to determine the nuclease degradation for each inhibitor.

Flow Cytometry

DU145 cells were treated with PEI alone, non-specific ODN (ns-ODN)/PEI (5 µM), T40214/PEI (5 µM), JG244/PEI (5 µM) or (T40214 + JG244)/PEI (2.5 + 2.5 µM for 24 hr, then washed with PBS and fixed with 75% ice cold ethanol for 30 min at room temperature. The cells were then re-suspended in staining buffer containing 10 µg/ml propidium iodide and 0.1% RNase. After 2-hr incubation at room

temperature, the cells were analyzed using a BD FACS flow cytometer with CellQuest acquisition and analysis software (Becton Dickinson, San Jose, CA). Gating was set to exclude cell doublets and cell clumps.

Establishing Xenograft Model

Athymic Balb-nu/nu 4–5 week old male mice were purchased from Charles River Laboratories. Approximately 5×10^6 DU145 cells in 200 µl of PBS were then injected subcutaneously into the right flank of each mouse. Also, 4–5 weeks old C57BL/6 male mice were purchased from the Jackson Laboratories. To study the effects of the GQ-ODN drugs on a mouse tumor model, 4×10^6 TRAMPC-2 cells in 200 µl of PBS were injected into the right flank of each mouse.

Formation of T402I4/PEI (or JG244/PEI) Complex

(i) G-rich ODN (T40214 or JG244) was dissolved in sterile H_2O at a concentration of 5 µg/µl. Then the solution was heated to 95°C for 10 min and gradually cooled to room temperature (about 3 hr or overnight). (ii) PEI was added to the T40214 (or JG244) solution to achieve a final complex concentration of 1 µg/µl of ODN with 0.25 µg/µl of PEI.

In vivo Measurement

(i) After the DU145 tumors reached ~150 mm³, mice were randomly assigned to five groups: Group 1 was used as controls without treatment; Group 2 received PEI treatment with PEI alone (2.5 mg/kg); Group 3 was treated with T40214/PEI (10 mg/kg + 2.5 mg/kg); Group 4 was treated with JG244/PEI (10 mg/kg + 2.5 mg/kg); Group 5 was treated with JG244/PEI and T40214/PEI (10 mg/kg + 2.5 mg/kg) alternately. Each group was composed of five mice. The first treatment was started as soon as the mice were divided into groups. These mice were treated by intravenous injection (IV) every 3 days for 31 days. Body weight and tumor size were measured every 3 days. Tumor volumes were calculated using the following formula ($a \times 0.5b^2$), where a equals the length and b equals the width of tumors. (ii) After TRAMPC-2 tumors reached ~150 mm³, the mice were randomly divided into five groups: Group 1 as control needed no treatment; Group 2 received PEI treatment; Group 3 was treated with ns-ODN/PEI (10 mg/kg + 2.5 mg/kg) as a control ODN; Group 4 was treated with paclitaxel (10 mg/kg), a chemotherapeutic drug as positive control; Group 5 was treated with T40214/PEI (10 mg/kg + 2.5 mg/kg); Group 6

was treated with (JG244 plus T40214)/PEI $((5 + 5)mg/kg + 2.5\ mg/kg)$. These groups were injected intraperitoneally every other day for 3 weeks. Tumor monitoring was done as described above. (iii) Following these treatments mice were sacrificed. The tumors were then harvested and stored at $-80°C$ for further studies. (iv) The unpaired two-sample t-test, $[t = (X_1 - X_2)/S_p{}^2(1/n_1 + 1/n_2))^{1/2}]$, was used to determine differences in tumor sizes between the control and the drug-treated groups.

Immunohistochemistry

DU145 prostate tumors were harvested from xenograft models, which had been untreated or treated with PEI alone, T40214, JG244, or the T40214, and JG244 combination treatment. These tumors were immediately frozen at $-80°C$, cryosectioned (5-µm), fixed in 3% buffered formalin for 15 min at room temperature and in methanol for 5 min at $-20°C$. Slides were washed 2×5 min in TBS plus 0.025% Triton X-100, subjected to antigen retrieval using citrate antigen buffer, and blocked in 10% normal serum with 1%BSA in TBS for 2 hr at room temperature. We then applied primary antibodies (p-Stat3 rabbit monoclonal (D3A7, Cell Signaling) at a dilution of 1:50 and VEGF mouse monoclonal (Santa Cruz) at a dilution of 1:100 and incubated overnight at 4°C. slides were rinsed 2×5 min in TBS 0.025% Triton and in 0.3% hydrogen peroxide in TBS for 15 min to block endogenous peroxidase activity. HRP-conjugated anti mouse antibody diluted 1:2,000 was used to detect the antigen–antibody complex. Then developed with chromogen-Metal Enhanced DAB (diamino-benzidine tetrachloride) solution. Finally sections were counterstained with hematoxylin, dehydrated, mounted, and observed. Sections were then evaluated on an Olympus BX40 light microscope and tumor immunostaining was scored on the percentage of immunopositive cells (0–100) multiplied by a staining intensity score (0/1/2/3), yielding scores of 0–300.

TUNEL Assay

Frozen tumors were sectioned 10–15 µm on glass slides pre-treated for electrostatic adherence. Slides were dried overnight at room temperature, rehydrated sequentially with 100%, 95%, and 70% ethanol and water, then fixed with 3.7% buffered formaldehyde for 10 min. Tissues were incubated with Cytonin for 30 min at room temperature. Apoptotic cells were detected by the Tumor TACS in situ apoptosis detection kit (Trevigen, Inc., MD) according to the manufacturer's instructions. Following quenching of endogenous peroxidase activity, slides were

incubated with labeling reaction mixture, and the reaction was stopped by stop buffer. Samples were covered with 50 μl of streptavidin-HRP solution. Finally color development was accomplished by immersing the slides in DAB solution for 5–10 min and counterstaining with methyl green. The slides were then mounted with glass coverslips and examined by light microscopy. Apoptotic positive cells in the tumors were quantified by counting the number of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) -positive cells in three randomly selected, non-overlapping regions using the light microscope.

RESULTS

Inhibitors of p-Stat3 and HIF-1α

Here, we have employed two agents T40214 and JG244 to target p-Stat3 and HIF-1α, respectively, and block DU145 prostate tumor growth. T40214 was determined to form a cylinder-like G-quartet structure composed of two G-quartets in the center and two T-G-T-G loop domains on the top and bottom [24,25], while JG244 was designed to form a chair G-quartet structure [23] (Fig. 1A). The different G-quartet structures cause T40214 and JG244 to selectively target p-Stat3 and HIF-1α/2α, respectively, [22,23,26]. For delivering the ODN into cells, PEI as a vehicle forms a complex with T40214 or JG244. The AFM results demonstrated that the size of ODN/PEI complex is about 25 nm (Fig. 1B) and the zeta potential showed the surface charge of ODN/PEI complex (Fig. 1C). Also, we performed the assays of delivering ODN/PEI complex in prostate cancer cells (DU145) using fluorescent labeled T40214 and JG244. The results demonstrated that T40214 and JG244 were well delivered inside cells and then entered into nucleus (Fig. 1D). This delivery system significantly increases the drug efficacy of these agents [21,23].

Suppressing the Levels of p-Stat3 and HIF-1α

The Western blots showed (Fig. 2A) that the expressions of HIF-1α and HIF-2α are pretty low in prostate cancer cells at 20% O_2 (Lane 1) due to rapid degradation by the ubiquitin-proteasome system. Under hypoxic conditions (1% O_2) compared with the high level of HIF-1α and HIF-2α in untreated cells (Lane 2), JG244 suppressed the expression of HIF-1α and HIF-2α in DU145 cells (Lanes 6–8). Densitomic analysis showed that the IC_{50}s of inhibition of HIF-1α and HIF-2α by JG244 are $\sim$ 2.4–2.7 μM, while T40214 showed no inhibition of HIF-1α and HIF-2α, as previously observed [23]. In contrast, T40214 inhibited IL-6-induced p-Stat3 activation in DU145 cells and its

IC_{50} is estimated as 3.3 μM, while JG244 did not show any inhibition of p-Stat3 (Fig. 2B). Also, T40214 inhibited IL-6-induced p-Stat3 activation in murine prostate cancer cells (TRAMP-C2) with IC_{50} of $\sim$4.1 μM (Fig. 2C). The results provide evidence that T40214 and JG244 selectively inhibit their respective targets, p-Stat3 and HIF-1α/2α, in both human and murine prostate cancer cells, as observed previously [22,23,26].

Resistance to Degradation in Serum

We tested mouse sera for resistance of G-quartet ODNs against the nuclease degradation, Fluorescent-labeled T40214 and JG244 mixed with serum, incubated at 37°C, and sampled from 0 to 96 hr and loaded in a denaturing polyacrylamide gel. Under UV light the gels showed the intact T40214 and JG244 in serum from 0 to 96 hr (Fig. 3A,B). After 96 hr more than 80% and 65% of T40214 and JG244, respectively, were still intact in serum (Fig. 3C). These analyses demonstrated that G-quartets T40214 and JG244 could keep drug activation in serum for a long time.

Induction of Apoptosis in Prostate Cancer Cells

Inducing apoptosis in cancer cells is an important factor for anti-cancer agents. Inhibition of Stat3 or HIF-1α/2α would greatly enhance apoptosis of cancer cells. To determine whether T40214 or JG244 can induce apoptosis in DU145, flow cytometry of propidium iodide-stained DU145 cells was employed to identify the population of sub-G1 (SG1) cells, which manifest DNA damage associated with apoptotic or necrotic cell death. The panels of cell cycles (Fig. 4) demonstrated that compared with the SG1 fraction of control cells (0.8%), no apoptosis was induced in the cells treated by PEI alone (1.2%) or ns-ODN/PEI (0.5%), which is a 20mer non-G-rich ODN used as a control. The percentage of cells in SG1 increased to 18.1% for T40214-treated and 21.2% for JG244-treated DU145 cells. However, the combined treatment mixing T40214 and JG244 together induced the percentage of cells in SG1 to 35.2%, showing that the treatment with combined two inhibitors together dramatically induced apoptosis in prostate cancer cells.

In vivo Anti-Tumor Efficacy

Tests of in vivo anti-tumor efficacy were performed by administration of the two agents, T40214 or JG244, every 3 days alone or alternately via IV. Nude mice with established ($\sim$150 mm^3) metastatic prostate tumors (DU145) were randomly assigned to five groups: (i) untreated; (ii) PEI (2.5 mg/kg);
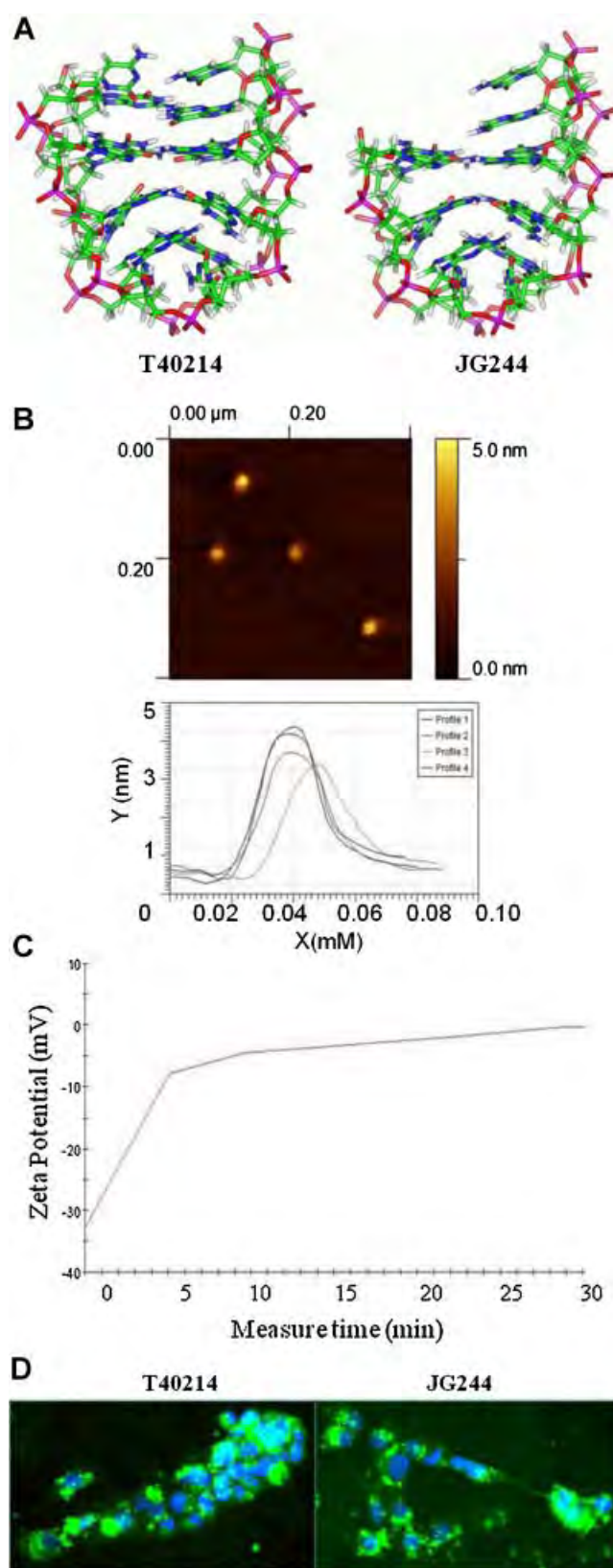
Fig. 1.   **A**: Molecular structures of T40214 and JG244. **B**: The mean diameter of T40214/PEI complex is about 25 nm. **C**: Zeta potential showed the surface charge of JG244/PEI complex. **D**: Microscopic photos demonstrated that T40214 and JG244 were delivered inside tumor cells (DU145) and nucleus.
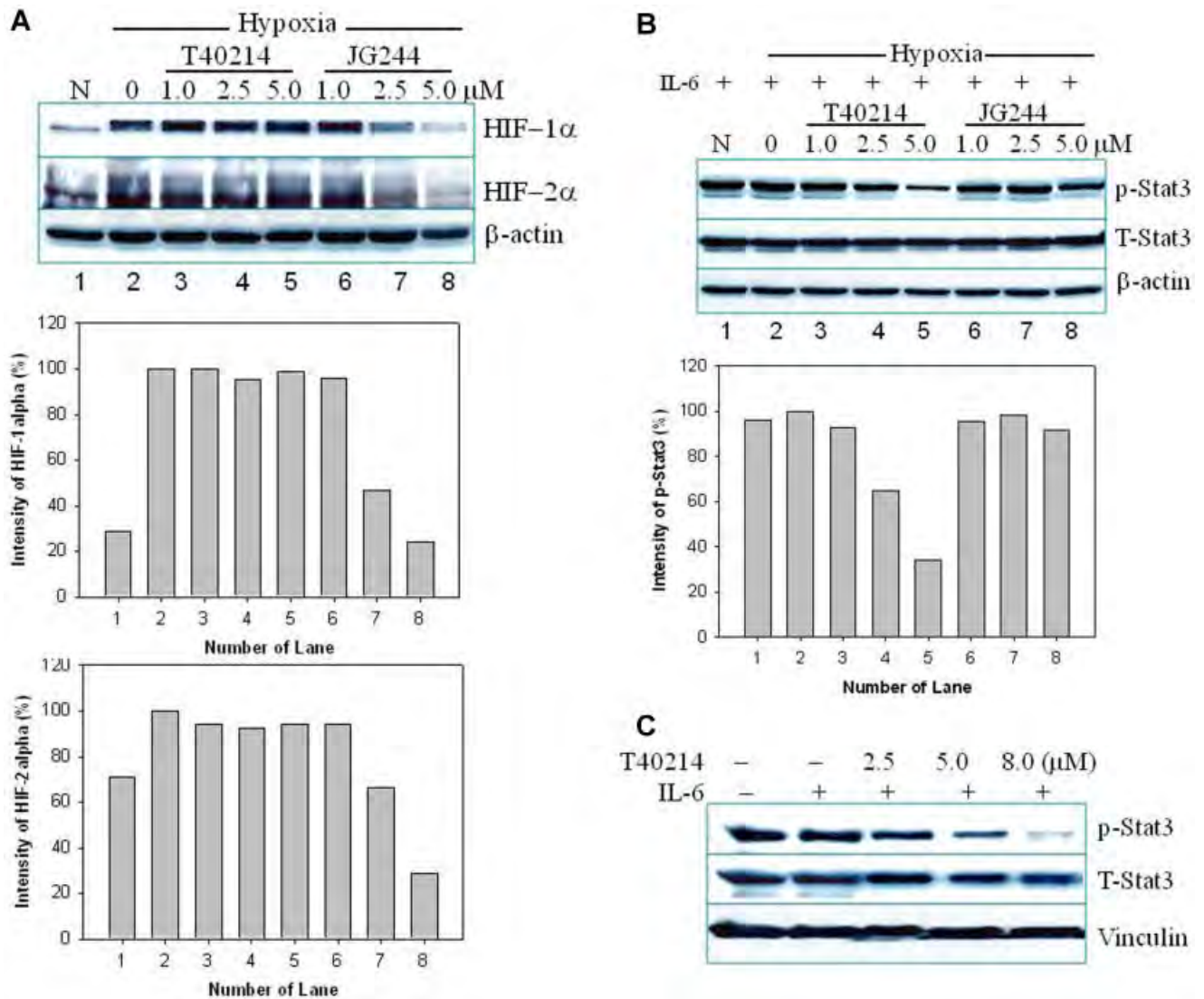
Fig. 2. **A,B**: JG244 and T402I4 inhibit the levels of HIF-1α/2α and p-Stat3, respectively, in DUI45 under hypoxia. The levels of HIF-1α/2α and p-Stat3 expressions were plotted versus number of lane, which corresponds to the number of Western blot lane. The band intensity (%) was calculated by compared with that of control sample (Lane 2). **C**: T402I4 inhibits phospho-Stat3 activity in murine prostate cancer cell (TRAMP-C2).

(iii) T40214/PEI (10 mg/kg + 2.5 mg/kg); (iv) JG244/PEI (10 mg/kg + 2.5 mg/kg); and (v) T40214/PEI and JG244/PEI (10 mg/kg + 2.5 mg/kg) alternately (e.g., T40214/PEI on day 1, day 7, day 13, etc., and JG244/PEI on day 4, day 10, day 17, etc.). Tumor volume and body weight were measured every 3 days. Over 31 days of drug treatment, the mean tumor volume in control mice and PEI-treated mice increased 5.8- and 5.2-fold (Fig. 5A) and mean body weights decreased 5–7%. The mean tumor volumes in mice treated with T40214/PEI and JG244/PEI alone increased 3.1 and 2.5-fold, respectively. The mean tumor volume in mice treated by alternating JG244/PEI and T40214/PEI increased only 1.5-fold.

The mean body weights of the drug-treated groups increased about 5–10%, showing that the two agents were not overtly toxic and were well tolerated by animals. Statistical analysis indicated that, compared with untreated tumors, the growth of the tumors treated by T40214 ($P < 0.05$) or JG244 ($P < 0.01$) or the combination of T40214 with JG244 ($P < 0.002$) were all significantly suppressed. Moreover, compared with T40214 alone, T40214 and JG244 given sequentially showed a significant increase in drug efficacy ($P < 0.05$).

To investigate whether the efficacy of T40214 and JG244 would be affected by the immune system, immunocompetent mice (C57BL/6) bearing murine
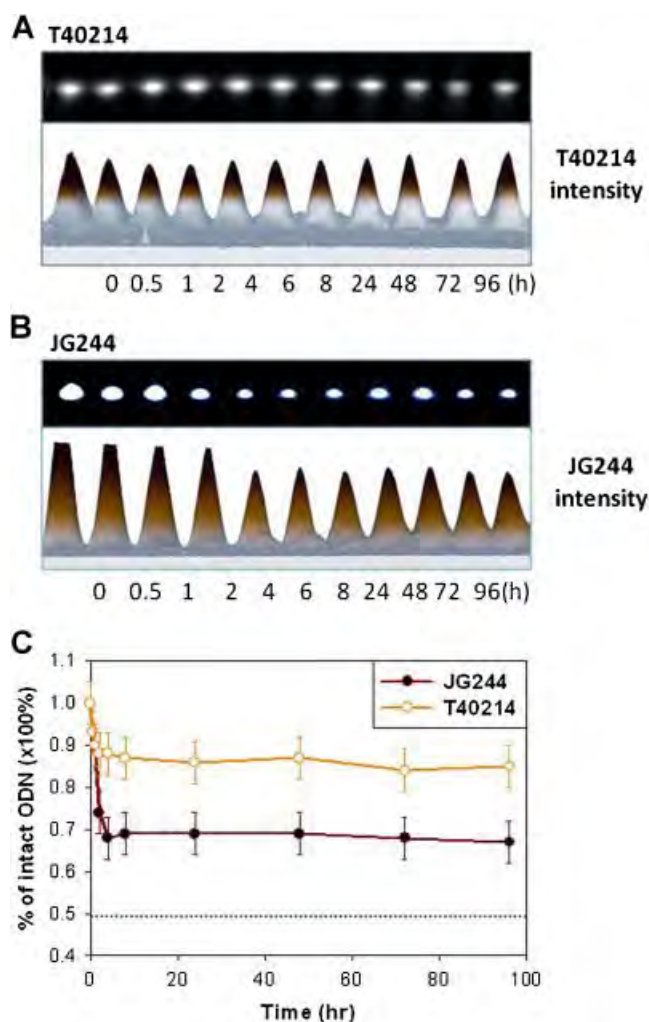
Fig. 3. Nuclease resistance assays. The gel shows how the intensities of T402I4 (**A**) and JG244 (**B**) varied with time from 0 to 96 hr in serum. **C**: Plot of % of intact ODN versus time provides the pharmacokinetic information on T402I4 and JG244 in serum.

prostate tumors (TRAMP-C2) were employed for in vivo drug tests. The sequence of Stat3 protein in murine cells is the same as that in human cells [27]. Also, a comparison between the murine and human HIF-1α protein sequence revealed 95%, 99%, and 83% identity in the bHLH, PAS, and variable domains, respectively [28]. When tumor size reached ~150 mm³, the mice were randomly assigned to various groups: (i) untreated (control); (ii) PEI alone (2.5 mg/kg); (iii) ns-ODN/PEI as a control oligo (10 mg/kg + 2.5 mg/kg); (iv) paclitaxel, a chemotherapeutic drug, as a positive control (10 mg/kg); (v) T40214/PEI (10 mg/kg + 2.5 mg/kg); and (vi) combination treatment mixing T40214 with JG244 [(5 + 5)mg/kg + 2.5 mg/kg]. The mice were treated every other day by intraperitoneal injection for 3 weeks (paclitaxel was given

every 4 days due to its toxicity). Over 21 days of drug treatment, the results (Fig. 5B) demonstrated that the mean tumor volumes of control, PEI-treated, ns-ODN/PEI-treated, and paclitaxel-treated mice increased 9.4, 9.4, 10.2, and 8.0-fold, respectively. The mean tumor volumes of the mice treated by T40214 alone and T40214/JG244 combined increased 5.6 ($P < 0.09$) and 3.1-fold ($P < 0.03$), showing that combining T40214 and JG244 together significantly suppressed the murine prostate tumor growth in immunocompetent mice.

Inhibition of p-Stat3 and HIF-1α in Prostate Tumors

To investigate the mechanism of inhibition of p-Stat3 or HIF-1α in prostate tumors, we harvested the DU145 tumors from nude mice and TRAMP-C2 tumors from C57BL/6 mice after drug treatment. The tumor Western blots demonstrated that compared with untreated tumors, tumors treated by PEI alone had no inhibition of either p-Stat3 or HIF-1α. T40214 significantly reduced p-Stat3 but not total Stat3 (T-Stat3), which was mainly composed of unphosphorylated Stat3, in DU145 prostate tumors (Fig. 6A). HIF-1α was highly expressed in the untreated prostate tumors, indicating substantial hypoxia inside the tumors since HIF-1α is mainly expressed in a hypoxic environment (Fig. 6B). JG244 completely inhibited the expression of HIF-1α in prostate tumors, while T40214 had no inhibition of HIF-1α. As a critical protein for angiogenesis, the level of VEGF in human prostate tumors was dramatically suppressed by both the Stat3 inhibitor T40214 and the HIF-1α inhibitor JG244. Stat3 was constitutively phosphorylated in murine prostate tumors (TRAMP-C2). Western blots (Fig. 6C) showed that PEI and ns-ODN has no inhibition of p-Stat3 and VEGF. Compared with T40214 alone, the combined treatment (T40214 + JG244) has a stronger activity to suppress the expressions of p-Stat3 and VEGF in TRAMP-C2 tumor. T40214 alone or the mixture of T40214 and JG244 did not inhibit expression of phospho-Akt (p-AKT) or total Akt (T-AKT) in the tumors.

Immunohistochemistry of p-Stat3 and VEGF

To further explore the mechanism of the combination treatment, we also employed immunohistochemistry to demonstrate the expression of p-Stat3 and VEGF in prostate tumors treated by the agents alone or in combination. The distribution of p-Stat3 and VEGF proteins in fresh tumor sections was detected by immunohistochemical staining of nucleus and cytoplasm, respectively (Fig. 6A,B). Phospho-Stat3 and VEGF were highly expressed in the
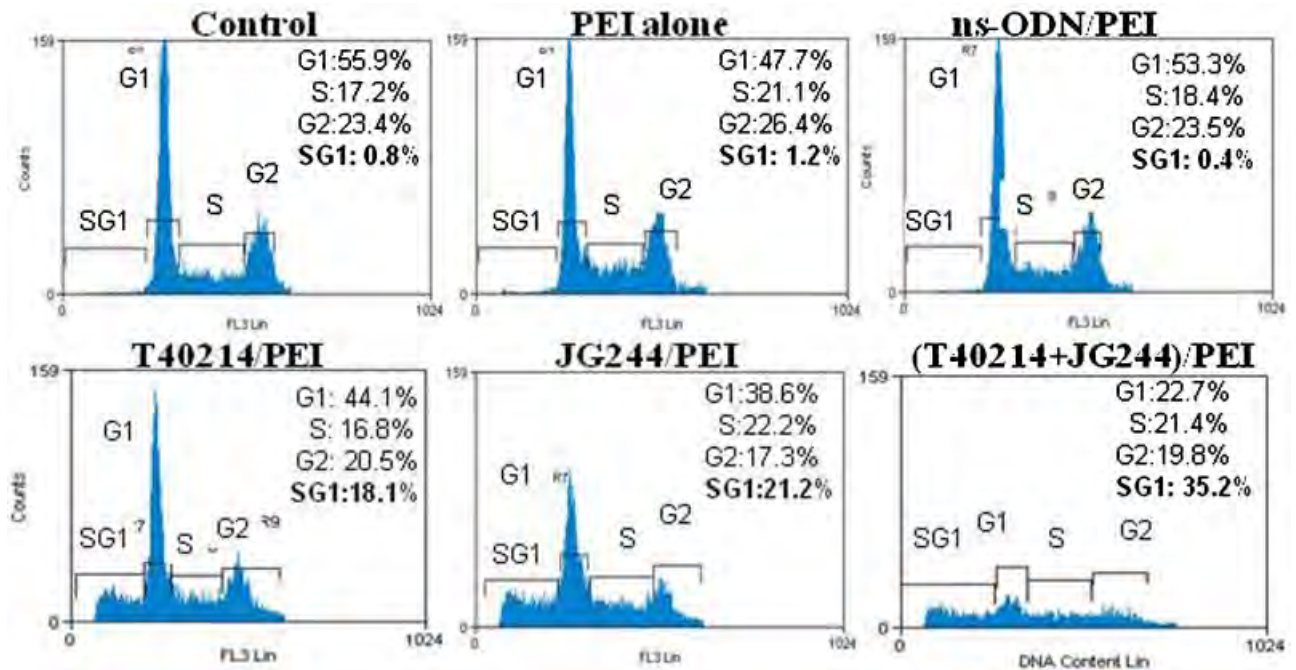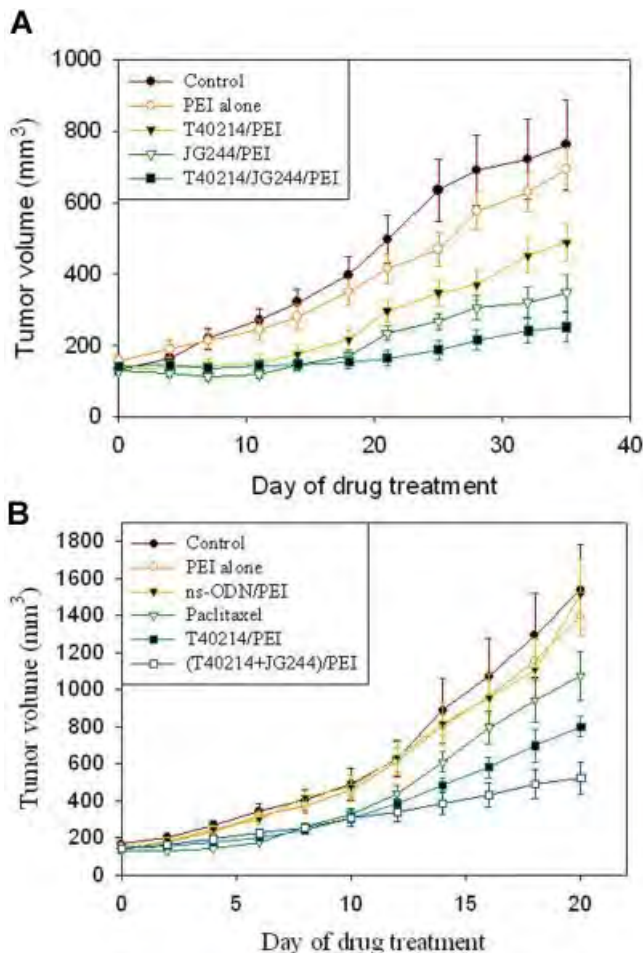
Fig. 4. PI staining showed that T402I4 and JG244 induced apoptosis in DUI45 prostate cancer cells subjected to hypoxia for 24 hr and the plot of % of SGI for each treatment. The SGI fraction consists of cells undergoing apoptosis.

untreated and PEI-treated tumors. Low expression of p-Stat3 was observed in the T40214 and T40214/ JG244-treated tumors. However, the tumors treated by JG244 showed moderate expression of p-Stat3, suggesting as expected that JG244 did not greatly inhibit p-Stat3 activation. VEGF expression was significantly suppressed in the tumors treated by T40214 or JG244 alone or combination. Inhibition of either p-Stat3 or HIF-1α dramatically suppressed the expression of VEGF in tumors, matching the observation in Figure 5.

Apoptosis Induced by T402I4 and JG244

TUNEL is a standard method for detecting DNA fragmentation by labeling the terminal ends of nucleic acids that result from apoptotic signaling cascades. Here we performed TUNEL assays in the harvested tumors using the TumorTACS In situ Apoptosis Detection Kit (Trevigen, Inc. MD) according to the manufacture's instruction. The kit-stain generated dark brown precipitates in cleaved DNA fragments, which correspond to apoptotic cells. The results from DU145 tumors demonstrated (Fig. 7C,E) that there are few apoptotic cells in untreated (3%) and PEI-treated

Fig. 5. In vivo drug efficacy. Plot of tumor volume versus day of drug treatment demonstrate greater efficacy of combination treatment in DUI45 human prostate tumors (A) and in TRAMP-C2 murine prostate tumor (B).
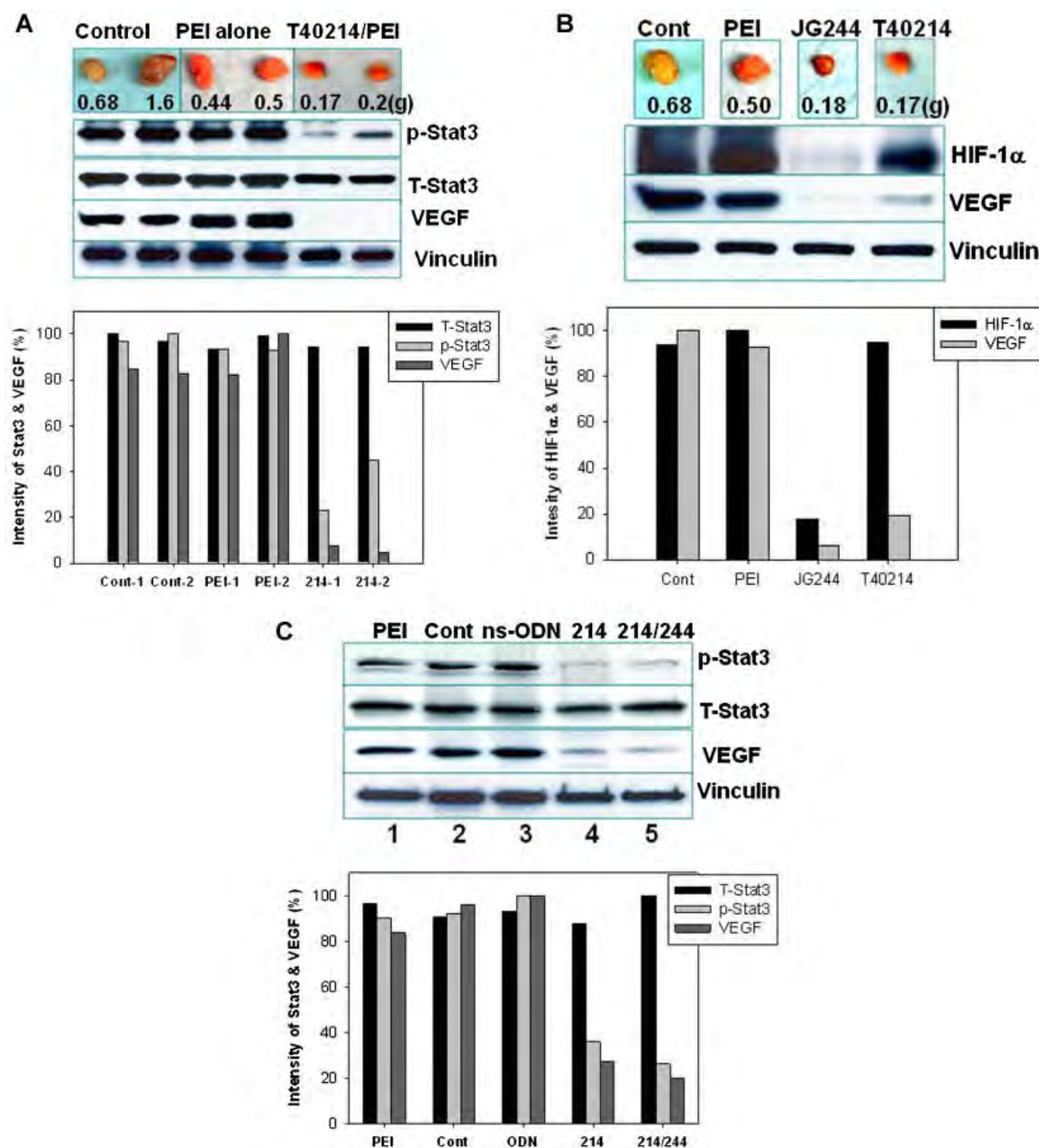
Fig. 6. Tumor Western blots. **A,B**: Western obtained from DUI45 tumors treated by control, PEI alone, T40214, and JG244, respectively. **C**: Western obtained from TRAMP-C2 tumors treated by control, PEI alone, ns-ODN (a control ODN), T40214, and T40214 plus JG244, respectively. The level of p-Stat3 expression was plotted versus number of lane, which corresponds to the number of Western blot lane. The band intensity (%) was calculated by compared with that of control sample (Lane 2).

tumors (8%), and many apoptotic cells in T40214/ PEI-treated (43%) or JG-244/PEI-treated tumors (39%). The combination treatment further dramatically promoted apoptosis in the prostate tumors (78%).

Similar results were also observed in TRAMP-C2 tumors. Compared with apoptosis induced by T40214 (35%), treatment with T40214 plus JG244 greatly enhanced apoptosis in the tumors (56%) (Fig. 7D,E).
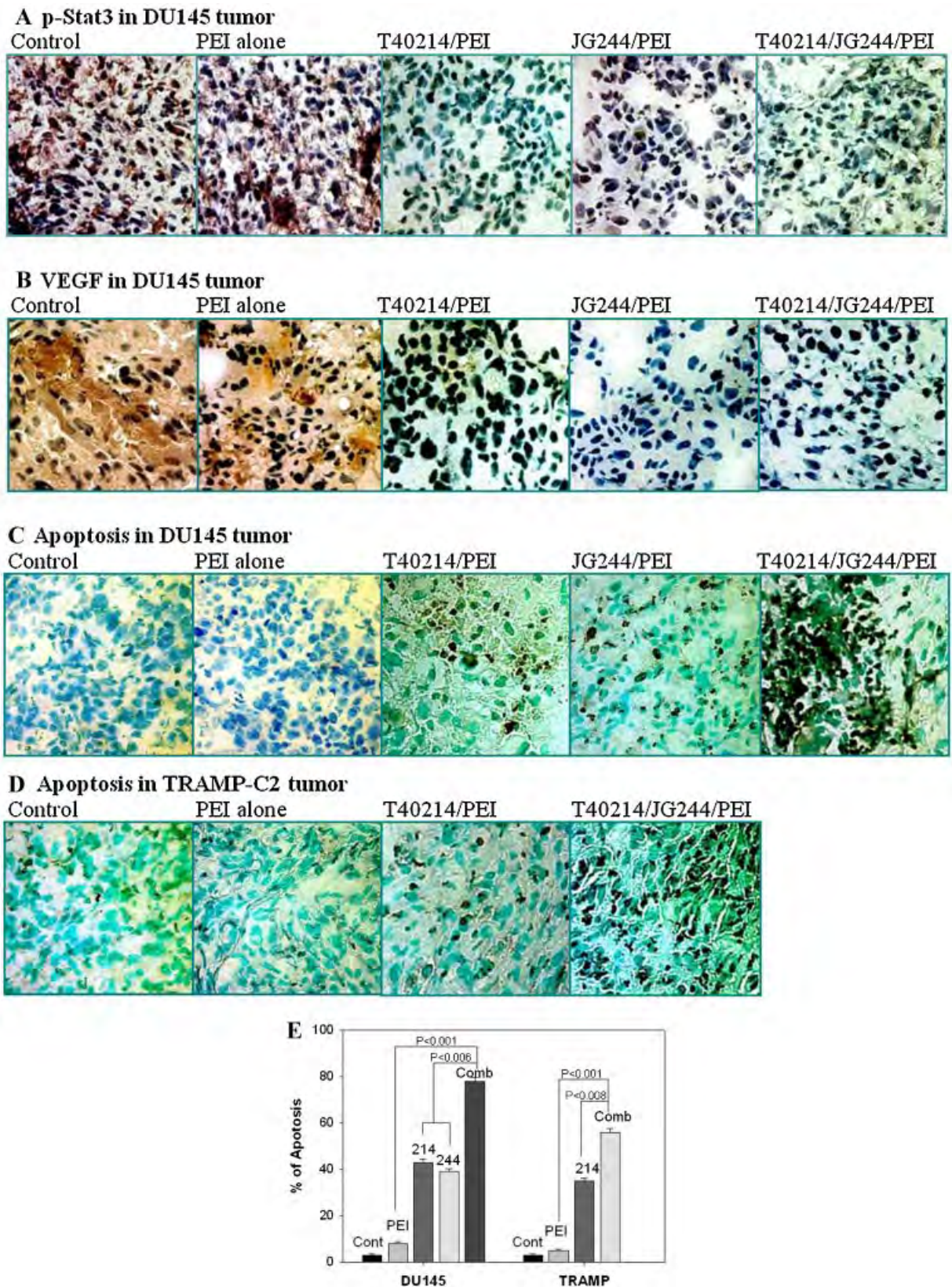
Fig. 7 Inmmunohistochemistry and TUNEL. Inmmunohistochemistry demonstrates the expressions of phosphorylated Stat3 (**A**) and VEGF (**B**) in prostate tumors (DUI45), including untreated tumors and tumors treated by PEI alone, T402I4, JG244, or the combination of T402I4 and JG244. The pictures show that p-Stat3 was stained brown in nuclei and cytoplasm and VEGF was stained brown in tumor tissues, respectively. The TUNEL results from DUI45 tumors (**C**) and TRAMP-C2 tumors (**D**) demonstrate that there are very few apoptotic cells in untreated tumors or PEI treated tumors. Apoptosis dramatically increased in the tumors treated by T402I4 combined with JG244. The TUNEL stain generates dark brown precipitates in the present of cleaved DNA fragments, which correspond to apoptotic cells. **E**: Plots of the percentage of apoptotic cells within prostate tumor xenografts assessed by TUNEL staining. Cont, control; Comb, combination treatment.

The Prostate

Statistical analysis indicated that in both DU145 and TRAMP-C2 tumors, the indexes of apoptotic cells between untreated and T40214/JG244-treated tumors or between PEI-treated and T40214/JG244-treated tumors are significant ($P < 0.001$). Compared with apoptotic cells induced by T40214 or JG244 alone, the combined treatment greatly increased apoptosis in DU145 ($P < 0.006$) and TRAMP-C2 tumors ($P < 0.008$). These results provided solid evidence that compared with the tumors treated by Stat3 inhibitor or HIF-1α inhibitor alone, combination treatment with inhibitors of both p-Stat3 and HIF-1α can significantly induce cell death in prostate tumors.

DISCUSSION

Recently, drug development has moved into targeting molecules based on their important biological function in cancer [29]. A selective inhibition of a target molecule greatly enhances therapeutic efficacy; however, it also results in unexpected consequences, such as development of drug resistance. Stat 3 and the hypoxia-sensing pathway are both up-regulated in prostate cancer. Stat3 has been identified as an important target for cancer therapy and is constitutively activated in 80% of prostate cancer [30]. HIF-1α has also been demonstrated to be over-expressed in many human cancers, including colon, breast, gastric, lung, skin, ovarian, prostate, renal, and pancreatic carcinomas [12]. Over-expression of HIF-1α in human cancers, which results from intratumoral hypoxia and genetic alterations, has been associated with poor prognosis and treatment failure in a number of cancers [12]. Targeting both phosphorylated Stat3 (p-Stat3) and HIF-1α would be expected to decrease the survival and angiogenic potential in tumor cells and could constitute a potent cancer therapy. Semenza group indentified several drugs that inhibit HIF-1, such as digoxin that inhibits HIFα protein synthesis [31] and acriflavine (ACF) that inhibits dimerization of HIF protein [32]. ACF, which was first reported 50 years ago [33], was identified as the most potent inhibitor of HIF-1α among the 3,120 drugs [32]. We have recently found that the novel inhibitor of HIF-1α/2α JG244 dramatically suppressed the growth of several tumors [23].

Here we proposed that inhibition of HIF-1α could vary the tumor microenvironment and reduce the influence of hypoxia, so that it could increase drug efficacy for collaborated drugs. To explore the hypothesis, we took our developed inhibitors: T40214 and JG244, to treat prostate tumors in xenograft models. Our in vivo results provided evidence that each inhibitor, T40214 and JG244, significantly suppressed tumor growth in prostate cancer xenograft

model. Alternately, treatment by the Stat3 inhibitor and the HIF-1α inhibitor had a much better drug efficacy than continuously giving either inhibitor alone (Fig. 4A). In order to test whether immuno-system will affect the drug efficacy of the combination treatment, we also evaluated the in vivo drug efficacy of T40214 and JG244 in inmuno-competent mice (C57BL/6) bearing a murine prostate tumor (TRAMP-C2) (Fig. 4B). Compared with the tumor growth in control, PEI-treated, or ns-ODN-treated mice, a mixture of T40214 and JG244 significantly suppressed murine prostate tumor growth and showed a much better drug efficacy than T40214 alone. These results demonstrated that inhibition of both Stat3 and HIF-1α together has a better drug efficacy than targeting either p-Stat3 or HIF-1α alone.

VEGF is a potent angiogenesis promoter and is over-expressed in numerous human cancers. VEGF enhances migration and proliferation, and reverses senescence of endothelial cells, and simulates blood vessel formation [34,35]. Previous studies showed that both Stat3 and HIF-1α can regulate VEGF expression and that inhibition of either Stat3 or HIF-1α transcription factors can significantly reduce VEGF expression [36,37]. HIF-1α and Stat3 are components of a large complex governing transcription of VEGF, and hypoxia-mediated VEGF expression requires binding both Stat3 and HIF-1α to the VEGF promoter for maximum induction in pancreatic and prostate carcinomas [38]. Our results (Figs. 5 and 6) showed that JG244 or T40214 not only selectively inhibited HIF-1α/2α or p-Stat3, respectively, but also completely blocked the expression of VEGF and significantly reduced angiogenesis potential in prostate tumor.

Enhancing apoptosis of cancer cells in tumors is an important strategy for cancer therapy. TUNEL results (Fig. 6C,D) demonstrated that each inhibitor, T40214 and JG244, greatly induced apoptosis of cancer cells in prostate tumors, consistent with the observation in prostate cancer cells in culture (Fig. 3). We show that the combination treatment demonstrated that JG244 cooperated with T40214 to dramatically increase apoptosis in prostate tumors and significantly suppressed tumor growth. Previous studies demonstrated that over-expression of HIF-1α contributes to resistance to both radiation therapy, and chemotherapy and is associated with treatment failure and increased patient mortality [39–41]. Our results showed that combination treatment targeting p-Stat3 and HIF-1α can increase tumor response as compared to either agent alone. Alternating the two agents provides a novel mechanism to reduce drug resistance and treatment failure.

Our results provide evidence that the combination treatment targeting p-Stat3 and HIF-1α could

improve tumor responses as compared to either agent alone. Given the two agents alternatively for treatment of prostate cancer may also be a mechanism to increase drug efficacy, reduce drug resistance and treatment failure. Although the mechanism of increasing drug efficacy by targeting both p-Stat3 and HIF-1α is not well understood yet, our results provide an information that inhibition of HIF-1α could have potential to reduce hypoxia-induced drug resistance and enhance drug efficacy for combined therapeutic agents (e.g., T40214) and that the combination treatment could make prostate cancer treatments more effective and improve survival of patients with metastatic disease. Whether combining with a HIF-1α inhibitor could promote therapeutic efficacy for other collaborated drugs in different solid tumors still needs further studies.

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REFERENCES

1. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernández-Luna JL, Nuñez G, Dalton WS, Jove R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999;10:105–115.

2. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS, Tweardy DJ. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth in vitro. J Clin Invest 1998;102:1385–1392.

3. Silvennoinen O, Schindler C, Schlessinger J, Levy DE. Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. Science 1993;261:1736–1739.

4. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415–1421.

5. Levy DE, Darnell JE, Jr. Stats: Transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3:651–662.

6. Becker S, Groner B, Muller CW. Three-dimensional structure of the Stat3beta homodimer bound to DNA. Nature 1998;394:145–151.

7. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Jr., Kuriyan J. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell 1998;93:827–839.

8. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474–2488.

9. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 2002;8:945–954.

10. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: Role of STAT3 in the tumour microenvironment. Nat Rev Immunol 2007;7:41–51.

11. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: A leading role for STAT3. Nat Rev Cancer 2009;9:798–809.

12. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–732.

13. Shi YH, Fang WG. Hypoxia-inducible factor-1 in tumour angiogenesis. World J Gastroenterol 2004;10:1082–1087.

14. Powis G, Kirkpatrick L. Hypoxia inducible factor-1alpha as a cancer drug target. Mol Cancer Ther 2004;3:647–654.

15. Hirota K, Semenza GL. Regulation of angiogenesis by hypoxia-inducible factor 1. Crit Rev Oncol Hematol 2006;59:15–26.

16. Roskoski R, Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. Crit Rev Oncol Hematol 2007;62:179–213.

17. Jing N, Li Y, Xiong W, Sha W, Jing L, Tweardy DJ. G-quartet oligonucleotides: A new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. Cancer Res 2004;64:6603–6609.

18. Jing N, Li Y, Xu X, Sha W, Li P, Feng L, Tweardy DJ. Targeting Stat3 with G-quartet oligodeoxynucleotides in human cancer cells. DNA Cell Biol ;22:685–696.

19. Jing N, Zhu Q, Yuan P, Li Y, Mao L, Tweardy DJ. Targeting signal transducer and activator of transcription 3 with G-quartet oligonucleotides: A potential novel therapy for head and neck cancer. Mol Cancer Ther 2006;5:279–286.

20. Weerasinghe P, Garcia GE, Zhu Q, Yuan P, Feng L, Mao L, Jing N. Inhibition of Stat3 activation and tumor growth suppression of non-small cell lung cancer by G-quartet oligonucleotides. Int J Oncol 2007;31:129–136.

21. Weerasinghe P, Li Y, Guan Y, Zhang R, Tweardy DJ, Jing N. T40214/PEI complex: A potent therapeutics for prostate cancer that targets STAT3 signaling. Prostate 2008;68:1430–1442.

22. Zhu Q, Jing N. Computational study on mechanism of G-quartet oligonucleotide T40214 selectively targeting Stat3. J Comput Aided Mol Des 2007;21:641–648.

23. Guan Y, Reddy KR, Zhu Q, Li Y, Lee K, Weerasinghe P, Prchal J, Semenza GL, Jing N. G-rich oligonucleotides inhibit HIF-1alpha and HIF-2alpha and Block Tumor Growth. Mol Ther 2010;18:188–197.

24. Jing N, Hogan ME. Structure-activity of tetrad-forming oligonucleotides as a potent anti-HIV therapeutic drug. J Biol Chem 1998;273:34992–34999.

25. Jing N, Xiong W, Guan Y, Pallansch L, Wang S. Potassium-dependent folding: A key to intracellular delivery of G-quartet oligonucleotides as HIV inhibitors. Biochemistry 2002;41:5397–5403.

26. Jing N, Sha W, Li Y, Xiong W, Tweardy DJ. Rational drug design of G-quartet DNA as anti-cancer agents. Curr Pharm Des 2005;11:2841–2854.

27. Pietra LD, Bressan A, Pezzotti AR, Serlupi-Crescenzi O. Highly conserved amino-acid sequence between murine STAT3 and a revised human STAT3 sequence. Gene 1998;213:119–124.

28. Luo G, Gu YZ, Jain S, Chan WK, Carr KM, Hogenesch JB, Bradfield CA. Molecular characterization of the murine Hif-1 alpha locus. Gene Expr 1997;6:287–299.

29. Hambley TW, Hait WN. Is anticancer drug development heading in the right direction? Cancer Res 2009;69:1259–1262.

30. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T, Falcone R, Fairclough R, Cantor A, Muro-Cacho C, Livingston S, Karras J, Pow-Sang J, Jove R. Constitutive activation of Stat3 in human prostate tumors and cell lines: Direct

inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002;62:6659–6666.

31. Zhang H, Qian DZ, Tan YS, Lee K, Gao P, Ren YR, Rey S, Hammers H, Chang D, Pili R, Dang CV, Liu JO, Semenza GL. Digoxin and other cardiac glycosides inhibit HIF-1alpha synthesis and block tumor growth. Proc Natl Acad Sci USA 2008;105:19579–19586.

32. Lee K, Qian DZ, Rey S, Wei H, Liu JO, Semenza GL. Anthracycline chemotherapy inhibits HIF-1 transcriptional activity and tumor-induced mobilization of circulating angiogenic cells. Proc Natl Acad Sci USA 2009;106:2353–2358.

33. Goldie H, Walker M, Graham T, Williams F. Topical effect of acriflavine compounds on growth and spread of malignant cells. J Natl Cancer Inst 1959;23:841–855.

34. Grothey A, Galanis E. Targeting angiogenesis: Progress with anti-VEGF treatment with large molecules. Nat Rev Clin Oncol 2009;6:507–518.

35. Punt CJ, Tol J. More is less – Combining targeted therapies in metastatic colorectal cancer. Nat Rev Clin Oncol 2009;6: 731–733.

36. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604–4613.

37. Wei D, Le X, Zheng L, Wang L, Frey JA, Gao AC, Peng Z, Huang S, Xiong HQ, Abbruzzese JL, Xie K. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. Oncogene 2003;22:319–329.

38. Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, Gallick GE. HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene 2005;24:3110–3120.

39. Chun YS, Adusumilli PS, Fong Y. Employing tumor hypoxia for oncolytic therapy in breast cancer. J Mammary Gland Biol Neoplasia 2005;10:311–318.

40. Fontanini G, Faviana P, Lucchi M, Boldrini L, Mussi A, Camacci T, Mariani MA, Angeletti CA, Basolo F, Pingitore R. A high vascular count and overexpression of vascular endothelial growth factor are associated with unfavourable prognosis in operated small cell lung carcinoma. Br J Cancer 2002;86: 558–563.

41. Ishigami SI, Arii S, Furutani M, Niwano M, Harada T, Mizumoto M, Mori A, Onodera H, Imamura M. Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 1998;78:1379–1384.